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(54) Preparation of clavulanic acid and its salts and esters.

(57) At least part of the assimilable carbon source is added to the culture medium when producing clavulanic acid by fermentation with a producing micro-organism. The clavulanic acid is readily obtained in good yield and purity, preferably as an ester or salt in a form suited for formulation as pharmaceuticals.

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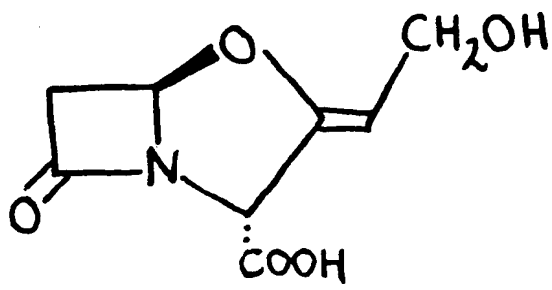
PREPARATION OF CLAVULANIC ACID AND ITS SALTS AND ESTERS

Technical Field

The present invention relates to the preparation of clavulanic acid, and pharmaceutically acceptable salts and esters thereof.

Clavulanic acid, (2R,5R,Z)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo-[3,2,0]-heptane-2-carboxylic acid, is a known compound of structure:



This compound, and its salts and esters, act as beta-lactamase inhibitors, and inhibit the action of beta-lactamases produced by both gram-positive and gram-negative organisms. They are therefore employed in pharmaceutical compositions to prevent inactivation of beta-lactam antibiotics. Furthermore, clavulanic acid is itself believed to have an anti-bacterial activity.

Prior Art

Clavulanic acid is produced from various strains of micro-organism, for example the strains belonging to the genus Streptomyces such as S. clavuligerus NRRL 3585 (USA Patent 4,110,165), S. jumonjinensis NRRL 5741 (British Patent 1,563,103), S. katsurahamanus IFO 13716 (Japanese Patent 83,009,579), and Streptomyces sp. P6621 FERM 2804 (Japanese Patent 55,162,993).

Modifications in the fermentation process have been used to increase the yield of clavulanic acid. For example, British patent 1,571,888 discloses that strict pH control to a range of 6.3 to 6.7 can markedly increase the yield.

More frequently, attempts to increase yield have involved the purification process. For exampl , clavulanic acid salts are generally more stable than the free acid. Consequently, salts such as alkali metal and alkaline-earth metal salts have been employed to enhance the extraction yield.

USA Patent 4,110,165 describes an extraction based upon the differential solubility between aqueous and organic phas s of clavulanic acid and its salts. Extraction is followed by several purifications in adsorbent material, and is thus lengthy and expensive.

The direct crystallization of clavulanic acid from the organic phase is therefore desirable. In Spanish Patent 494,431, clavulanic acid is directly crystallized from the organic phase by addition of an amine such as t-butylamine, followed by conversion to a potassium salt by exchange with a base.

The lithium salt is also considered a useful intermediate in the preparation of clavulanic acid, due to its low solubility and its easy conversion to salts or esters, acceptable for pharmaceutical formulations. For example, USA Patent 4,490,294 discloses the reaction of a solution of clavulanic acid with an aqueous ionic lithium compound, thereby to yield lithium clavulanate. British Patent 1,543,563 discloses another route to lithium clavulanate, utilizing the adsorption of filtered broth in granulated carbon, an ion-exchange resin treatment and crystallization of lithium clavulanate.

Crystallization from isopropanol of lithium clavulanate, after direct adsorption of the fermentation broth in the resin, has also been suggested. Such a process is difficult and gives poor yields.

Hence, the fermentation and purification methods of the available technology have produced impure clavulanic acid in yields which are relatively low.

Description of the Invention.

According to a first aspect of the present invention there is provided a method for the production of clavulanic acid by fermentation in a culture medium by a producing micro-organism, in which method at least part of the assimilable carbon source is added during the fermentation.

Without being limited by theory, it is believed that the controlled addition of the carbon source leads to the absence of catabolic regulation. Regardless of theory, it is observed that a marked improvement can be obtained by taking care to prevent the carbon level from being relatively high at any time during the fermentation.

Preferred Embodiments

For preference, the level of assimilable carbon source is kept below 2%, better still below 1% (the percentages are on a weight/volume basis for solid carbon sources, and volume/volume for liquid carbon sources). Correspondingly, it is preferred to add the carbon source in low amounts to the production medium, either batchwis or continuously. Any addition of carbon sourc is pref rably in an amount of not more 1%, or better still not more than 0.5%, relative to th medium. Indeed, the carbon level in the medium is pref rably maintained at below 0.25%, such as <0.15%.

Any assimilable carbon source can be employed, including a polyol or a carbohydrate, but more especially a chemically defined carbon source (that is, a carbon compound of chemically known structure). The defined carbon source typically has a molecular weight of below 500, and suitably comprises up to 12 carbon atoms in the molecule, such as in a mono- or di-saccharide, or a linear polyol. Examples of such compounds include maltose and glycerol. In an alternative preferred form of the present invention, the assimilable carbon source is a dextrin, starch or dextrinized starch. More generally, the carbon source is usually water soluble or water miscible.

The use of S. clavuligerus, especially S. clavuligerus NRRL 3585, is currently preferred. For such micro-organisms the preferred carbon sources include maltose, glycerol, dextrins, and optionally dextrinized starchs. Glucose, fructose, sucrose and lactose are not so readily assimilable by S. clavuligerus, and are therefore less preferred for such micro-organisms.

The carbon source can be incorporated continuously or intermittently, preferably subject to the constraint that the level of carbon source does not rise at any time above 2%. For instance, incorporation can be continuously, using constant metering of slight amounts of carbon source in to the medium. The continuous addition is suitably effected at a rate of around 0.01

to 0.1%/hour, more preferably from about 0.5 to 1.5%/day, particularly about 0.05%/hour. In an alternative mode, incorporation is effected batch-wise in accordance with a set protocol. For example, intermittent addition can be at spaced intervals ranging normally from every minute to every 6 hours. The daily addition may total 0.25% to 1.5%, although the optimum daily amount ranges from 0.5 to 1.5%%.

The incorporation need not necessarily follow a constant pattern. Thus, an incorporation of 0.5 or 1%/day can be maintained over the first 60 hours, followed by 1 or 0.5%/day, respectively, for the rest of the fermentation. Furthermore, the amount of carbon source initially present in the medium upon inoculation preferably ranges from 0% to 2%, more preferably from 0% to 0.5%, and most preferably from 0% to 0.05%.

It is preferable to add the carbon source continuously or intermittently in a sterilized manner, and to provided means to ensure and maintain this sterility. Furthermore, it is preferable that solid carbon sources are added in solution in water. Liquid carbon sourc s may be added directly or may be diluted appropriately.

Th f rmentation m dium can contain other suitable cultur c mponents, including an organic nitrogen sourc . Such nitrog n sources can comprise oleaginous s ed, optionally defatted meals, protein hydrolysates or

extracts. Mineral salts (particularly chlorides, sulphates, carbonates or phosphates) and defoaming agents of mineral or organic origin can added where appropriate, together with other known medium components.

The optimum fermentation temperature is ordinarily between 24 and 30°C, particularly from 26 to 28°C, but the invention is not tied to such temperatures since fermentation can take place above or below these limits. The fermentation is typically effected on a batch system taking from 1 to many days, more usually from 24 to 240 hours, and especially 100 to 200 hours.

The most suitable fermentation vessel for the invention is a conventional aerobic fermentation vessel provided with means for agitation and aeration. The volume of such vessels can vary for instance from 1 liter and 225 m3. The working volume is preferably from 25 to 75% of the total capacity.

In working up the broth, the solids are preferably first removed from the fermentation broth by filtration or centrifugation. It is also preferred that the broth is acidified to a pH of 1 to 3, preferably around pH 2, and clavulanic acid extracted by adding a water-immiscible solvent, with the two phases being separated for example by centrifugation, thereby giving the clavulanic acid in the water-immiscible phase.

The present invention further extends to a new process for obtaining clavulanic acid as its lithium salt, and for optionally converting the lithium clavulanate to other salts or to esters of clavulanic acid.

Thus, more specifically, in accordance with a second aspect of the present invention, there is provided a process for the purification of clavulanic acid, the process comprising:

(i) mixing the dissolved impure clavulanic acid with dissolved lithium 2-ethylhexanoate, the acid and the salt each being preferably dissolved in a water-immiscible solvent, more preferably the same solvent;

(ii) isolating lithium clavulanate; and

(iii) optionally converting the lithium salt to another salt or to an ester.

By such a process it is possible to obtain both enhanced yield and purity. The process is of general applicabilty to the purification of clavulanic acid fermentation broths, but it is especially suited to purifying extracts produced in accordance with the process of the first aspect of this invention. As mentioned above, such a fermentation can readily lead to a solution of impure clavulanic acid in a water-immiscible solvent, suited for use in th step (i).

The prefered organic solvents are ethyl acetate, methyl acetate, amyl acetate, methyl isobutyl ketone or n-butyl alcohol, with n-butyl alcohol being most preferable. Extraction of the broth is preferably conducted at a temperature in the range 4 to 10°C, with the purpose of minimizing losses due to decomposition of the clavulanic acid.

Following extraction, crystallisation may be carried out by the addition of a solution of lithium 2-ethylhexanoate, suitably prepared in known manner. For preference, the concentration of the clavulanic acid in the organic solvent is more than 10 mg/ml. Crystallization is best performed at low temperatures, normally between 4 and 10°C. Since lithium clavulanate crystallizes with one quarter of a molecule of water, it is recommended that crystallization is performed in the presence of a small concentration of water, such as 2 to 4% in the organic solvent. Excess lithium 2-ethylhexanoate is preferably used. Addition can take place slowly under stirring and cooling. The stirring is best maintained for a period of time after all lithium 2-ethylhexanoate has been added.

The resultant lithium clavulanate can then be further processed, and is preferably filtered through a glass plate, washed with isopropanol, acetone and ether, and dried under vacuum.

The lithium clavulanate may be recrystallized. The recrystallization can be carried out by adding ato aqueous lithium clavulanate an excess of an organic solvent in which the solubility of the lithium salt is very low, preferably isopropanol. Alternatively, and more preferably, a concentrated solution of another lithium salt is added to aqueous lithium clavulanate to cause the recrystallization. Suitable lithium salts include organic or inorganic lithium salts having high solubility in water. Lithium chloride is most preferable. Suitably, the concentration of the lithium salt solution is more than 50%, and sufficient solution is added so that the final concentration is more than 25%. Addition of the lithium salt solution preferably takes place slowly, while the lithium clavulanate solution is stirred.

The recrystallized lithium clavulanate may be further purified, for instance it can be separated by filtration, washed with acetone and dried under vacuum, thereby obtaining a yield of more than 80% and a purity above 85%.

Lithium clavulanate prepared in accordance with this invention can be converted to other salts by ion-exchange procedures, using cationic exchange resins, in the form of th desired cation, preferably sodium or potassium, and luting with water. Furthermore, esters can be made in accordance with known procedures.

For use as an alternative to lithium salt formation, or more often in addition thereto, the present invention further present a series of improvements in the extraction of clavulanic acid using ion-exchange resins which permit a higher exchange resin adsorption capacity. Examples of suitable resins are described, for instance, in UK Patent 1,543,563. The resin is preferably a weak basic ion exchange resin of the IRA-68 type and is preferably in the form of the acetate.

The fermentation broth suitably has an initial activity of more than 800 µg/ml of clavulanic acid, in order to obtain a good crystallization of the lithium clavulanate, after adsorption of the broth in the ion exchange resin, elution thereof with a lithium salt and subsequent crystallization for example from isopropanol.

Before adsorption of the broth in the resin, it is best to effect a clarification.

In accordance with a third aspect of the present invention, the fermentation broth is treated with an aggregating agent to aggregate the mycelium.

Aggregation of the mycelium permits a better filtration. A suitable aggregating agent is the filter coadjuvant Praestol (trademark of Bayer AG, Germany). The coadjuvant is preferably added to the broth at a concentration of from 0.1 to 0.5%, and the

mix stirred for about 15 minutes before filtration. The filtration itself is suitably effected by passage through a bed of inorganic filter material such as Dicalite (typically about 6% w:v) in a Buchner funnel (Dicalite is a trademark of Dicalite Ltd). If the first volumes of filtration are not well clarified, Refiltration through the same Dicalite prelayer can be employed. The broth can also be clarified, after filtration, by centrifugation (typically 30 minutes at 10,000 rpm).

Another improvement of this invention is an increase in the adsorption capacity of the ion-exchange resin achieved by deproteinization of the filtered broth.

The deproteinization is preferably effected by acidification, usually to around pH 4, or by treatment with a precipitating solvent such as acetone.

In an embodiment of the invention, the pH of the filtered broth is adjusted to about 4.0 with mineral acid, such as hydrochloric or sulphuric acid. Once the pH has been adjusted, the supernatant is adsorbed in the resin. The broth is preferably first allowed to stand, and any turbidity eliminated by centrifugation. To prevent product losses due to th pH of around 4, the mixture can be brought back to about pH 6 before adsorption on the resin. The adsorption capacity of the resin in the form of acetate at a pH of

6 is increased in this manner. Preferably the ratio of filtered broth:resin (v/v) is not more than 10:1

The filtered broth can also be deproteinized by treatment with a precipitating solvent, such as acetone. In an embodiment of the present invention, one volume of broth is mixed with about one volume of acetone. For preference, the broth is stirred for 5 to 10 minutes with acetone, allowed to stand for 20 minutes, and the precipitate separated by centrifugation or filtration. The broth thus clarified is suitable for its adsorption in the resin. The acetone can be recoverable by distillation under reduced pressure before adsorption of the broth. Whether the acetone has been removed or not, the filtered broth can be adsorbed in the resin at a ratio of from 10:1 to 12:1 (initial volume of filtered broth:volume of resin) without considerable losses.

Crystallization of lithium clavulanate from the eluates of ion exchange resins has previously led to formation of gums and in some cases a low purity. To minimize this problem, it is preferred to wash or pre-elute the resin with 0.5% sodium chloride and then with water, before elution with the lithium salt, such as 5% lithium chlorid .

Crystallization of the lithium clavulanate can be further improved by treatment of the eluate with

acetone. The eluate of the resin is stirred for example with two volumes of acetone and allowed to stand for 15 minutes. The precipitate is separated by centrifugation to allow crystallization of the lithium clavulanate from isopropanol, after the acetone has been eliminated.

More generally, the present invention can readily give clavulanic acid, its salts and esters, in a form suited for formulation as pharmaceuticals.

The present invention is illustrated in non-limiting manner by the following examples.

EXAMPLE 1

The following medium was prepared:

fishmeal	2.0	g
glycerol	1.5	g
soluble starch	1.5	g
calcium carbonate	0.2	g
distilled water to	100	ml

The pH was adjusted to 7, and 40 ml aliquots were plac d in 250 ml Erlenmeyer flasks and sterilized.

The medium was th n seeded with a suspension of spores of S. clavuligerus NRRL 3858 and incubated for 2 days at

28°C under agitation. The culture was employed to seed, to 2%, 500ml of the same medium in a 2-liter Erlenmeyer flask. Culturing was also carried out on a rotary shaker at 28°C for 2 days.

500 ml of the culture thus prepared were used to seed a 340-liter stainless steel vessel fitted for agitation, aeration and temperature control. The vessel contained 100 liters of a sterilized medium of the following composition:

	%
soybean meal	3
corn dextrin	3
soybean oil	0.5
KH_2PO_4	0.1
defoamer (ucon)	0.005
tap water to final volume	

The seeded medium was cultured at 28°C, receiving air with stirring for 45 hours. Thereafter, a further 340-liter vessel was seeded, to 7%, with 150 liters of the same medium. Incubation took place at 28°C for 30 hours, under stirring and aeration.

Upon termination, this culture was used to seed, to 7%, a clavulanic acid production medium contained in a tank. The tank had a total capacity of 800 liters, and 425 liters of the following medium were added:

soybean meal	1.25	%
peanut meal	1.25	%
distiller's dried grain	0.5	%
KH_2PO_4	0.1	%
defoamer (ucon)	0.005	%
tap water to final volume		

Sterilization took place at 121°C for 20 minutes, after adjusting the pH to 6.7. Glycerol was added intermittently every hour as follows:

time (h)	rate (ml/hr)	total glycerol
0 to 12	180	0.5%
13 to 96	190	3.7%
97 to 160	100	1.5%

Fermentation was conducted at 26°C under stirring and aeration. After 160hrs the level of clavulanic acid was high, at 1403 µg/ml.

EXAMPLES 2 AND 3

COMPARATIVE EXAMPLES 1 AND 2

The effect of adding the gylycerol gradually during fermentation was compared directly with the effect of including all the glycerol at the start of the fermentation.

The following table illustrates the results:

example*	% glycerol		clavulanic acid	
	initial	addition	112 h	160 h
E2	0	5.7	819	1403
E3	1	4.8	402	669
CE1	3.5	0	359	273
CE2	5.5	0	198	199

* E, Example; CE, Comparative Example

A substantial improvement in production is noted wh n the assimilable carbon source is incorporated during the fermentation.

EXAMPLE 4

40 liters of the same fermentation production medium as in Example 1 were seeded with the same inocolum in a 75 litre fermenter. A sterile, aqueous solution of 50% maltose was then added continuously using a continous addition diaphragm pump, at the rate of 800 ml daily (1% maltose/day) for 112 hours, at which time the broth was collected for extraction. The fermenter was agitated and aerated in conventional fashion. The conc ntration of the clavulanic acid observed in the broth after 112 hours was 1,424 µg/ml.

EXAMPLE 5

7.5 Liters of n-butanol were added to 7.5 liters of filtered broth from Example 1, and the pH was adjusted to 2 with cold 4N sulphuric acid. The mixture was centrifuged to 16,000 g, thereby separating the two phases, aqueous and butanolic. The butanol phase was concentrated under reduced presure at 35°C to a volume of 200 ml.

In this way, a solution of clavulanic acid in 200 ml of n-butanol was obtained with a concentration of 26.7 mg/ml. 5 ml of water were added, giving a moisture level of 2.52%. To this solution, under stirring, were added 100 ml of 10% solution of lithium 2-ethylhexanoate in n-butanol. The total volume was concentrated to 150 ml, it was stirred for 2 hours at 5°C and filtered through a filtering plate. The crystalline form was washed with 100 ml of isopropanol, 100 ml of acetone and 100 ml of ether, followed by drying at 40°C under vacuum, to give 7.57 g of the product, purity 54%, moisture 3.2% and 23.5% ash (as lithium sulphate).

EXAMPLE 6

10 g of lithium clavulanate obtained as in Example 5 were dissolved in 50 ml of water, adding thereto 75 ml of a solution of 50 w/v of lithium chloride slowly under

stirring. Crystallization started, and the mix was stirred for half an hour at 5°C and filtered through a glass flask, washed with acetone and dried under vacuum at 40°C, obtaining 5.27 g of product, yield 84% amd purity 80%.

Physical characteristics of the recrystallized lithium clavulanate

IR (cm^{-1}, Nujol (trade mark)): 3420, 3010, 1765, 1680, 1620, 1400, 1340, 1325, 1300, 1220, 1200, 1130, 1100, 1060, 1050, 1020, 990, 970, 950, 900, 880, 850, 730, 708.

$^{20}[\alpha]_D$ = 451 (c=145 µg/ml in water)

UV λ_{max} = 259 (c=10 µg/ml in O.IN NaOH)

(extinction molar coefficient, ϵ = 19,451)

lithium content (by absorption) = 3.3%

ash = 26.5%, as lithium sulphate.

EXAMPLE 7

8000 ml of a total fermentation broth (activity 1280 µg/ml of clavulanic acid) prepared in accordance with Example 1 or 4, were stirred for 15 minutes with filtration coadjuvant, such as 0.5% Praestol. Th broth was mixed with Dicalite at a ratio of 6:1 (w/v) and filtered carefully through filtering paper in a Buchner flask. After the broth had be n filtered and clarified, the pH was adjusted to 4.0 for deproteinization. The precipitate was separated by

centrifugation. The supernatant was adsorbed at pH 6.0 in a basic IRA-68 resin, in a column having a v/v filtered broth/resin ratio of 10:1. This resin was in the form of the acetate, pH 4.0.

Once the broth was adsorbed, the resin was washed with 0.5% sodium chloride and then with water. The clavulanic acid was eluted as the lithium salt using lithium chloride. The eluate of the resin (510 ml, activity: 4,500 µg/ml of clavulanic acid) was treated with two volumes of acetone, and the precipitate separated by centrifugation. The supernatant was mixed with 5 volumes of isopropanol, and again the precipitate separated by centrifugation. The supernatant was concentrated, allowing the lithium salt to crystallize at 4°C for 24 hours.

1182 mg were obtained, yield 11.5% and purity 96.92%/

EXAMPLE 8

A total fermentation broth (5,000 ml, activity: 1396 µg/ml of clavulanic acid) was filtered as described in Example 7.

The filtrate was mixed with one volume of acetone under continous stirring, allowing it to settle for 15 to 20 minutes. The sediment was removed by centrifugation and the acetone was taken off on a rotary evaporator.

The clarified broth was adsorbed in an IRA-68 ion-exchange resin as described in Example 7 (broth:resin ratio of 10:1 v/v). In this same manner the resin was washed and the lithium clavulanate was eluted to be crystallized from isopropanol. The yield was 20%. The eluate was separated into two fractions. The first fraction gave 650 mg, purity 69.3% and total yield 9%. The second fraction of the eluate gave 770 mg, purity 98% and total yield 11%.

The 69.3% pure crystallized lithium clavulanate contained 5% lithium and 35.4% ash as lithium sulphate. The lithium clavulanate was recrystallized from 55% lithium chloride, to give a product with the following characteristics:

ε = 15,908 (in 0.1 N NaOH)

lithium content = 3.6%

ash = 25%, as lithium sulphate

purity = 95%

IR (Nujol, cm^{-1}) 3430, 3010, 1760, 1680, 1615, 1375, 1300, 1210, 1130, 1105, 1065, 1050, 1030, 995, 980 ,950, 900, 885, 855, 740, 710.

EXAMPLE 10

A total broth (4,000 ml with 300 µg/ml of clavulanic acid) was treated with Praestol and filtered as in Example 7. The broth was clarified at pH 6.0 and passed over a mixed column of IRA-68 and XAD-2 resin

mixed at a ratio of 40:1 (volume of filtered broth: volume of resin).

The eluate of the pre-column containing practically 100% of the initial clavulanic acid activity was adsorbed in a column at a broth:resin ratio (v/v) of 10:1. The resin was washed with 0.5% sodium chloride and then with water. The lithium clavulanate was eluted with lithium chloride. Once the clavulanate was crystallized, as described in Example 7, 181 mg of the first product were obtained, purity 95.3% and yield 15.08% The physical properties were similar to those of the product of Example 7.

EXAMPLES 11 to 16

Different treatments were tried as part of adsorption of the broth in IRA-68 resin, acetate form. For Example 11, the broth was filtered and subjected to resin treatment was adopted. For Example 12, the resin was first washed with sodium chloride solution. For Example 13, the broth was filtered with coadjuvant. For Example 14, deproteinization of the broth at pH 4 was effected in addition to the procedure of Example 13. For Example 15, deproteinization of the broth with acetone was effected in addition to the procedure of Example 13. For Example 16, post treatment of the eluate with acetone was effected in addition to the procedure of Example 14.

example	exhaustion	wash	eluate	yield	purity
11	30-40	0-5	10-15	5	variable
12	30	5-10	10-15	5	50-80
13	0-7	0-1	50	5-10	60-80
14	0-2	0	60-70	10-20	80
15	0-2	0	70	20-22	80-90
16	0-2	0	70	20-25	90-95

The yields refer to the first collection of lithium clavulanate crystals.

The optimum process involves a filtration in the presence of a coadjuvant, a deproteinization at pH 4.0 and treatment with acetone prior to crystallization.

EXAMPLES 17 AND 18

The process of the preceding Examples can be further improved by first passing the filtered broth over a pre-column of IRA-68 or IRA-68 mixed with XAS-2, then deproteinizing or not at pH 4.0 or with acetone and the direct adsorption in the ion-exchange resin, as already indicated. For Example 17, a pre-column treatment was included in the procedure of Example 13, while for Exampl 17 a deproteinization at pH 4 and post-treatment with acetone was included in the procedure of Example 18.

example	exhaustion	wash	eluate	yield	purity
13	0-7	0-1	50	5-10	60-80
17	5	0	40-50	10-20	80
18	0-2	0	50	20-30	90-95

An increase in the adsorption capacity of the resin and better yield and purity of the end product was obtained in the Examples 17 and 18.

CLAIMS

1. A method for the production of clavulanic acid by fermentation in a culture medium by a producing micro-organism, in which method at least part of the assimilable carbon source is added during the fermentation.

2. A method according to claim 1, wherein the level of assimilable carbon source is kept below 2%.

3. A method according to claim 2, wherein the carbon level is maintained at below 0.25% during the productive fermentation.

4. A method according to any of claims 1 to 3, wherein the carbon source is incorporated continuously or intermittently at a rate of from 0.5 to 1.5%/day.

5. A method according to claim 4, wherein the carbon source is incorporated at a rate of about 0.05%/hour.

6. A method according to any preceding claim, wher in the clavulanic acid is purified from solution using th

steps of

(i) mixing the dissolved impure clavulanic acid with dissolved lithium 2-ethylhexanoate;

(ii) isolating lithium clavulanate; and

(iii) optionally converting the lithium salt to another salt or to an ester.

7. A method according to any preceding claim, wherein the clavulanic acid is purified using an ion-exchange resin.

8. A method according to any preceding claim, wherein the fermentation broth is treated with an aggregating agent to aggregate the mycelium.

9. A method according to any preceding claim, wherein the fermentation broth is filtered and deproteinized.

10. A method for the production of clavulanic acid, the method involving the steps of

(a) providing a culture medium lacking at least some assimilable carbon;

(b) inoculating the culture medium with a producing micro-organism;

(c) initiating fermentation of the culture medium by the micro-organism;

(d) adding assimilable carbon during th fermentation;

(e) obtaining a fermented broth containing clavulanic acid, and

(f) purifying the clavulanic acid from the broth.

European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 85 30 7783

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	JOURNAL OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY, vol. 31, 1981, pages 127-134, Society of Chemical Industry, Blackwell Scientific Publications, GB; G. LILLEY et al.: "Control of the production of cephamycin C and thienamycin by streptomyces cattleya NRRL 8057"Pages 127,129,132	1	C 12 P 17/18
A	CHEMICAL ABSTRACTS, vol. 98, no. 19, 9th May 1983, page 382, no. 159073w, Columbus, Ohio, US; C.H. PAN et al.: "Methyl oleate-based fermentation medium for cephalosporin C production", & DEV. IND. MICROBIOL. 1982, 23, 315-23 * Abstract *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 P 17/00 C 07 D 498/00 C 12 P 35/00 C 12 P 37/00
A	CHEMICAL ABSTRACTS, vol. 91, no. 11, 10th September 1979, page 603, no. 89456c, Columbus, Ohio, US; J.S. LEE et al.: "Effects of carbon sources and other process variables in fed-batch fermentation of penicillin", & MISAENGMUL HAKHOE CHI 1978, 16(1), 21-9 * Abstract *	1	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	31-01-1986	CHOULY J.

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